



## Contents of nitrate, nitrite and the occurrence of bacteria in fermented cooked parboiled rice and their potential ingestion in the diet

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### ABSTRACT

*In our region, people use the fermented cooked parboiled rice as their breakfast. Therefore, our present study was conducted to analyse the levels of nitrate, nitrite and the occurrence of bacteria in four different brands of rice which are found in Northern part of Sri Lanka especially in Jaffna Peninsula. Freshly and fermented cooked parboiled rice extracts were prepared for each variety separately and its nitrate and nitrite concentration was determined using spectrophotometric method. Naturally occurring bacteria were isolated from both parboiled rice and characterized based on morphological, physiological and biochemical characters. The results showed that higher levels of nitrate and nitrite were presence in the fermented cooked parboiled rice rather than the fresh parboiled rice. As well as, the bacterial population was higher in fermented cooked parboiled rice than fresh ones after 24 hours of incubation. Hence, among the isolated bacterial genera, Bacillus spp. was the wide spread bacteria in both parboiled rice. Daily intake estimates indicate that the nitrite content seems to satisfy the current acceptable daily intake, but nitrate does not satisfy the current acceptable level. Nevertheless, except in brand D, amount of nitrate and nitrite concentration in most of isolated fermented cooked parboiled rice were found in lower level due to the addition of additives such as pickled lime, onion, chilli. Therefore it is concluded that the presence of nitrite, nitrate and, Bacillus spp. (nitrate reducing bacteria) in fermented cooked parboiled rice may provide favourable environment to produce nitrosamine invivo and invitro in human beings. However, further studies should be done for the quantitative estimation of nitrosamine and also to characterize the bacteria in species level.*

**Key words**-fermented cooked parboiled rice, spectrophotometry, nitrosamine, Bacillus spp.

### INTRODUCTION

Nitrate and nitrite are occurred naturally in soil, water, and foods. But, nowadays foods have more amounts of nitrate and nitrite contents. Because, the agronomic practice of large application of nitrogenous fertilizers to obtain higher yields and improper disposal of human and animal waste may lead to accumulation of nitrate in food plants [1] and also nitrate and nitrite are used as food additives. In addition, accumulation of nitrate and nitrite due to the conversation of organic nitrogen compounds by microbial action.

In previous studies showed, the relationship of nitrate and nitrite are possible to form carcinogenic nitrosamine in foods for human. [1,2]. Humans are exposed to a wide range of N-nitroso compounds from diet, tobacco smoking, work place and drinking water, which are the major source in general population [2, 3]. The nitrosamines are formed endogenously or exogenously. Smoked preserved foods, foods subjected to drying by additives, curd meat product are main source for exogenous nitrosamine. The endogenous nitrosamine formed from nitrate, nitrite and nitrogen organic compound [3]. Presence of Nitrite in food can undergo nitrosation reactions in the gastrointestinal tract and bladder with amines and amides to give rise to N-nitroso compounds [2, 3, 4, 5]. Some of the N-nitroso compounds are carcinogenic and induce cancers in a variety of organs, including stomach, colon, bladder, lymphatics and hematopoietic system in many different animal models [5].

In our region many old people as well as others prefer to eat fermented cooked parboiled rice as their breakfast. The fermented cooked parboiled rice is made from fresh cooked parboiled rice due to the addition of water and allows it to stand for next day. The presence of microorganism such as *Arthrobacter*, *Bacillus*, *Pseudomonas*, *Mycobacterium*, *Nocardia*, *Streptomyces* and Soil fungi convert nitrate to nitrite, nitric oxide, nitrous oxide and nitrogen gas, degrade protein to secondary amines and create appropriate environment for formation of nitrosamine [3]. Some of people prefer to eat fermented cooked parboiled rice with onion, chilli and pickled lime. Using of these additives such as onion, green chilli and pickled lime shows antimicrobial activity [8]. Therefore, present study was undertaken to evaluate the concentration of nitrate, nitrite and bacteria which are found in four different types of cooked parboiled rice from Jaffna Peninsula.

## MATERIALS AND METHODS

### Preparation of fresh rice extract

Four different brands of parboiled rice were selected for this study from Jaffna peninsula. 500 ml of distilled water was boiled at 100°C in aluminum pot, and 100 g of brand A parboiled rice was placed into it to make it as soft form. The mixture was allowed to cool at room temperature. Cooked rice was homogenized with the help of pestle and 200 ml of distilled water was added to it. The mixture was stirred well for five minutes and the extract was collected by the filtration method. Other three brands extracts were also prepared by the above procedure separately. Nitrate and nitrite content were measured by using spectrophotometer.

### Preparation of fermented cooked parboiled rice extract

200 ml of distilled water was added to the cooked brand A parboiled rice. The mixture was homogenized and allowed at room temperature for nearly 20 hours. Then the extract was prepared by same method used for the preparation of fresh rice extracts. Nitrate and nitrite content of filtrate were taken using the spectrophotometer. The above procedure was repeated to other rice varieties as well.

### Preparation of fermented cooked parboiled rice extract with food additives

Pickled lime (1.5 g) was added to the 50 ml of Brand A fermented extract and mixture of Onion (1.0 g) and green Chilly (0.5) were added to another 50 ml of above extract, which were allowed to one hour. Then amount of nitrate and nitrite were measured. The procedure was repeated for other brands.

Table 1 Nitrate and Nitrite level of fresh and fermented cooked parboiled rice at room temperature

Samples	Mean value of nitrate content (mg/kg)		Mean value of nitrite content (mg/kg)	
	Fresh cooked parboiled rice	Fermented cooked parboiled rice	Fresh cooked parboiled rice	Fermented cooked parboiled rice
Brand A	866.7	1053.3	7.9	9.7
Brand B	1026.7	1786.6	9.0	13.5
Brand C	1000.0	1433.3	10.3	15.1
Brand D	1606.7	2466.7	8.9	11.2

Table 2: Estimated daily intake of nitrate and nitrite from fermented cooked rice

Brands	Estimated daily intakes in mg/kg body weight per day for fermented cooked rice	
	Nitrate	nitrite
Brand A	5.3	0.05
Brand B	9.0	0.07
Brand C	7.2	0.08
Brand D	12.4	0.06

Table 3. Nitrate and nitrite level in fermented cooked parboiled rice after the addition of food additives

Sample	Nitrate (mg/kg)	Nitrite (mg/kg)
Brand A	1040	8.4
Brand A Pickled Lime	640	7.4
Brand A+ Onion+ Chilli	940	7.4
Brand B	930	11.1
Brand B+ Pickled lime	810	8.1
Brand B Onion + Chilli	330	7.2
Brand C	1040	16.0
Brand C+ Pickled Lime	720	12.6
Brand C+ Onion + Chilli	980	11.4
Brand D	1160	12.2
Brand D+ Pickled Lime	1360	16.4
Brand D+ Onion + Chilli	1300	17.4

Table 4: Morphological characteristics of bacterial isolates from fresh and fermented cooked parboiled rice samples

Isolates code	Colony morphology				
	Colour	Form	Edge	Elevation	Surface
FBA1	Dull white	irregular	dentate	flat	smooth
FBA2	Dull white	irregular	dentate	flat	smooth
FBA3	yellow	filamentous	filamentous	flat	smooth
FBB1	Dull white	irregular	dentate	flat	smooth
FBB2	Dull white	irregular	dentate	flat	smooth
FBC1	Dull white	Spreading type			
FBC2	Dull white	irregular	dentate	flat	smooth
FBD1	Dull white	irregular	dentate	flat	smooth
FBD2	Dull white	irregular	dentate	flat	smooth
FBD3	Dull white	irregular	dentate	flat	rough
RBA	white	Circular	entire	flat	smooth
RBB1	Dull white	Rhizoid	Rhizoid	flat	smooth
RBB2	Dull white	Toruloid	lobate	flat	smooth
RBB3	Light yellow	Circular	entire	convex	shiny
RBC1	Dull white	irregular	dentate	flat	smooth
RBC2	Dull white	irregular	dentate	flat	smooth
RBD1	Dull white	irregular	dentate	flat	smooth
RBD2	Dull white	irregular	dentate	flat	smooth

Table 5: physiological and biochemical characteristic of bacterial isolates from fresh cooked parboiled rice samples

SAMPLE CODE	SHAPE OF CELL	GRAMS STAIN	ENDOSPORE	MOTILITY	CATALASE FERMENTATION	GLUCOSE FERMENTATION	NITRATE REDUCTION	FERMENTATION TEST	OXIDATION TEST	OXIDASE TEST	AEROBIC GROWTH	ANAEROBIC GROWTH
FBA1	Rod	Purple Gram Positive	Oval Central, terminal	(+)	3x	(+) No gas	(-) No gas, (+) Zn dust	(+)	(+)	(-)	(+)	(+)
FBA2	Rod	Purple (+)	Oval Central, terminal	(+)	3x	(+) No gas	(-) No gas, (+) Zn dust	(+)	(+)	(-)	(+)	(+)
FBA3	Rod	Purple (+)	Oval Central	(+)	2x	(-) No gas	(-) Gas production, (-) Zn dust	(-)	(+)	(+)	(+)	(+)
FBB1	Rod	Purple (+)	Oval central, sub terminal	(+)	3x	(+) No gas	(+) No gas	(+)	(+)	(-)	(+)	(+)
FBB2	Rod	Purple (+)	Oval Central	(+)	3x	(+) No gas	(+) No gas	(+)	(+)	(-)	(+)	(+)
FBC1	Rod	Pink Gram Negative	(-)	(+)	x	(+) No gas	(+) No gas	(+)	(-)	(-)	(+)	(+)
FBC2	Rod	Purple (+)	Oval Central	(+)	2x	(+) No gas	(+) No gas	(+)	(+)	(-)	(+)	(+)
FBD1	Rod	Purple (+)	Oval Central	(+)	x	(+) No gas	(+) No gas	(-)	(+)	(-)	(+)	(+)
FBD2	Rod	Purple (+)	Oval central, sub terminal	(+)	x	(+) No gas	(+) No gas	(-)	(-)	(-)	(+)	(+)
FBD3	Rod	Purple (+)	(-)	(+)	x	(+) No gas	(+) No gas	(+)	(+)	(-)	(+)	(+)

FB: Fresh cooked parboiled rice Brand

### Isolation of bacteria

10 g of the sample (fermented brand A rice) was transferred into the 90 ml sterile distilled water in Duran bottle under aseptic condition and the sample was crushed by a surface sterilized glass rod. The bottle was shaken well and allowed it for sedimentation. The supernatant was taken with sterile pipette and serial dilution was carried out until  $10^{-3}$  dilution. This procedure was repeated to all brands separately. Then 0.1ml of each extracts were transferred into the centre of sterilized nutrient agar plates separately and spread uniformly by using sterile glass spreader. After that plates were incubated at  $37^{\circ}\text{C}$  for 24-48 hours. After the incubation of 24 hours, the numbers of different types of colonies were counted in each plate. Finally pure cultures were prepared from the each culture plates. The isolated bacteria were characterised based on morphological, physiological, biochemical characters and nitrate test was done.

**Reduction of nitrate test:** A loop full of inoculum was transferred into sterilized nitrate broth. Control was also maintained. These were incubated at 37°C for 2 days. Then these were tested with one ml of nitrate reagent '1' and followed by one ml of nitrate reagent '2'

Reagent 1 – 0.8% sulphanilic acid in 5N acetic acid.

Reagent 2 – 0.5% dimethyl  $\alpha$ - naphthylamine in 5N acetic acid

After the addition of two reagents, red colour formation indicated as positive result. Negative result tubes were tested with zing pellets, for the presence of nitrate and Gas formation in Durham's tube was also noted.

**Table 6: physiological and biochemical characteristic of bacterial isolates from fermented cooked parboiled rice samples**

SAMPLE CODE	SHAPE OF CELL	GRAMS STAINING	ENDOSPORE	MOTILITY	CATALASE	GLUCOSE FERMENTATION	NITRATE REDUCTION	FERMENTATION TEST	OXIDATION TEST	OXIDASE TEST	AEROBIC GROWTH	ANAEROBIC GROWTH
RBA	Rod	Pink Negative (-)	(-)	(+)	x	(+) Gas production	(+) No gas	(+)	(+)	(-)	(+)	(+)
RBB1	Rod	Purple (+)	oval central, sub terminal	(+)	2x	(+) No gas	(+) No gas	(+)	(-)	(-)	(+)	(+)
RBB2	Rod	Purple (+)	Oval Central, terminal	(+)	x	(+) No gas	(+) No gas	(+)	(+)	(-)	(+)	(+)
RBB3	Rod	Pink Gram Negative	(-)	(+)	x	(-) No gas	(+) No gas	(+)	(-)	(-)	(+)	(+)
RBC1	Rod	Purple (+)	Oval Central	(+)	x	(+) No gas	(+) No gas	(+)	(-)	(-)	(+)	(+)
RBC2	Rod	Purple (+)	Oval Central	(+)	x	(+) No gas	(+) No gas	(+)	(-)	(-)	(+)	(+)
RBD1	Rod	Purple (+)	Oval Central	(+)	x	(+) No gas	(+) No gas	(+)	(+)	(-)	(+)	(+)
RBD2	Rod	Purple (+)	Oval central, sub terminal	(+)	x	(+) No gas	(+) No gas	(+)	(+)	(-)	(+)	(+)

RB: Fermented cooked parboiled rice Brand ; (+) - Positive result; (-) - Negative result; X - Low activity; 2X - Moderate activity  
3X - High activity

**Table 7: list of identified genera of isolated bacteria in samples and results of nitrate reduction test**

Samples Code	Genera
FBA1	<i>Bacillus</i> spp.
FBA2	<i>Bacillus</i> spp.
FBA3	<i>Bacillus</i> spp.
FBB1	<i>Bacillus</i> spp.
FBB2	<i>Bacillus</i> spp.
FBC1	<i>Enterobacteria</i> spp.
FBC2	<i>Bacillus</i> spp.
FBD1	<i>Bacillus</i> spp.
FBD2	<i>Bacillus</i> spp.
FBD3	<i>Listeria</i> spp.
RBA	<i>Enterobacteriaspp</i>
RBB1	<i>Bacillus</i> spp.
RBB2	<i>Bacillus</i> spp.
RBB3	<i>Enterobacteriaspp</i>
RBC1	<i>Bacillus</i> spp.
RBC2	<i>Bacillus</i> spp.
RBD1	<i>Bacillus</i> spp.
RBD2	<i>Bacillus</i> spp.

#### Nitrate and Nitrite level of fresh and fermented cooked parboiled rice at room temperature

In this study, Nitrate level varied from 866.7 to 1606.7 mg/kg for cooked parboiled rice and 1053.3 to 2466.7 for fermented cooked rice. On the other hand, Nitrite level showed a range between from 7.9 to 11.6 and 9.7 to 15.1 for fresh cooked parboiled rice and fermented cooked rice respectively. These results show in Table 1.

According to the World Health Organization (WHO), Accepted Daily Intake (ADI) of nitrate is 0.0 to 3.7mg/kg of body weight nitrate per day whereas; nitrite is 0.0 to 0.13mg/kg of body weight nitrite per day [9]. But unfortunately our work revealed that the fermented cooked rice contained higher level of nitrate level rather than ADI level. However nitrite is very low amount compared to ADI data. This estimated daily intake of nitrate and nitrite from fermented cooked rice shown in Table 2.

Overall, mean value of nitrate and nitrite content were found as a lower level in fresh cooked parboiled rice compared with fermented cooked parboiled rice.

The variation in nitrate and nitrite level in different brands of fermented cooked rice may be due to the difference in temperature, moisture, type and amount of microorganism, concentration of nitrate and nitrite in fresh rice, quality of water and nature of rice.

#### **Nitrate and nitrite level in fermented cooked parboiled rice after the addition of food additives**

According to the Table 3, except Brand D, the nitrate and nitrite levels were decreased due to the addition of additives.

These results enriched the knowledge of antimicrobial activity of additives [8], which are more sensitive towards the gram positive bacteria [8]

#### **Bacterial isolates from fresh and fermented cooked parboiled rice samples**

In serial dilution techniques, the population of microorganism in fermented cooked parboiled rice was higher than the fresh ones (Figure 1 and Figure 2) Bacterial isolates were characterised based on morphological, physiological and biochemical characters (Table 4-7)

In this study 18 isolates of bacteria were identified from the cooked fresh and fermented cooked parboiled rice. 15 isolates of them were Gram positive where most of them were become under the genera of *Bacillus* spp. previous study also proved that the *Bacillus* spp. is the mostly available genera in rice [7,10]

The presence of *Bacillus* spp.(nitrate reducing bacteria, our result also give evidence) in fermented cooked parboiled rice, which facilitates the enzymatic reduction of nitrate to nitrite and N-oxides. They also degrade proteins to secondary amines and create a favourable environment for the formation of nitrosamines- exogenous [3].

### **CONCLUSION**

Present study concluded that the nitrate and nitrite contents of cooked parboiled rice varied to each brand. However the levels of nitrate and nitrite in fermented parboiled rice and the amount of bacterial colonies were also found to be higher in fermented product. Nevertheless, the addition of food additives reduced the amount of both nitrate and nitrite concentration in most of the fermented parboiled rice, except in brand D. Hence, *Bacillus* spp.were found to be as the wide spread bacteria in the both tested sample. Hence, further studies should be carried out for the quantitative estimation of nitrosamine and also to identify the bacteria in species level. Further nitrate and nitrite contents of the water and other food items that make up the diet add up to the total intake. The findings in this study therefore need further verification by more detailed studies, which should include a wider volume of food materials and varieties of parboiled rice.

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